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THE CIRCULATING HEMOCYTE POPULATION OF THE MOLE-CRAB *EMERITA (= HIPPA) ASIATICA* MILNE EDWARDS

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The circulating hemocyte populations of crustaceans differ from species to species (Yeager and Tauber, 1935). The magnitude of interspecific variation in the hemocyte population of crustaceans appears to be very wide, ranging from 286 cells/mm^3 (*Astacus fluviatilis*, Hardy, 1892) to 128,000 cells/mm^3 (*Callinectes sapidus*, Sawyer, Cox and Higginbottom, 1970). In part, such variations could be due to diluents used to prevent clotting of the hemolymph and clumping of the cells (Stewart, Cornick and Dingle, 1967). Yeager and Tauber (1935) also reported intraspecific variation in hemocyte populations of crustaceans. The intraspecific variations in hemocyte populations cannot be entirely explained by the differences in techniques used in the counting procedures. In *Eriocheir sinensis*, Bauchau and Plaquet (1973) have shown variations in total hemocyte count in relation to different physiological conditions of the animal. In addition, environmental factors may also influence the hemocyte population in crustaceans. Dean and Vernberg (1966) have shown that total hemocyte counts in *Uca pugilator* acclimated to 30° C are higher than those of crabs acclimated to 10° C. Although these studies suggest that variations in the circulating hemocyte population may result from a number of variables including techniques, physiological factors and environment, the mechanism underlying such variations has not hitherto been elucidated.

The purpose of the present investigation is to evaluate the obvious factors which may influence circulating populations of hemocytes of the mole-crab *Emerita asiatica*. This would provide, (first), a firm basis for the determination of normal hemocyte counts in crustaceans generally, as well as for future experimental investigations on the animal; and (secondly), elucidation of the mechanism underlying the maintenance of hematological equilibrium in crustaceans.

Materials and Methods

Specimens of the mole-crab *Emerita asiatica* were collected from the sandy shores of Madras beach, opposite to University buildings. Collections were made twice a day, between 8 AM and 1 PM and between 2 PM and 3 PM. Animals were used for analysis within six hours. In the laboratory, animals were kept in rectangular glass troughs containing sand and sea water obtained from the area of collection. Only female crabs in the intermolt stage were used in this study. Prior to every analysis, crabs were immersed in filtered sea water at room temperature (28–30° C) for few minutes. The carapace length was measured, and the water content of the hemolymph was determined. Blood samples were collected by cutting the first walking leg on one side of the animal. Care was taken to avoid mixing fine sand grains or sea water with the blood sample. Total hemocyte

counts (THC) were made in a Neubauer (improved double) hemocytometer. Care was taken not to touch the chamber or coverslip, as it is known that fingerprints may also affect the counts (Dacie and Lewis, 1968). The coverslip was placed horizontally on the chambers and pressed gently on the sides. The hemolymph was allowed to fill the chamber in capillary motion about 5 sec after cutting the leg. The second chamber was filled subsequently after filling the first chamber.

In most of the previous investigations (except Drach, 1939 and Hoffmann, 1969), the hemolymph was diluted either with anticoagulants or with antiagglutinants (or with saline or Ringer's solutions containing one of the above), in order to avoid gelification of plasma and agglutination of cells. In this study neither of these diluents were used. It was reported earlier (Ravindranath, 1975a) that the gelification of plasma in *Emerita asiatica* occurs at room temperature 150 sec after amputating the leg. It may be noted that within about 5 sec of amputation of the appendage, the hemolymph fills the chamber and the cells settle within 60 to 100 sec. Plasma gelification which may occur subsequently does not alter the position in which cells have settled. However, as reported earlier (Ravindranath, 1975a), alteration of cells occur after about 5 min under normal conditions at 30° C. This does not interfere with counting as the cells are clearly visible even after alteration. Agglutination of cells in the hemocytometer was observed only when total count exceeded 20,000 cells/mm^3, which is a very rare condition in normal, agile and healthy animals. Counts were made in all squares of both upper and lower chambers of the hemocytometer. The hemocyte count/mm^3 of hemolymph was calculated from each hemocytometer count and recorded separately.

For studying the effect of size on the cell population, counts were taken in normal, intermolt animals belonging to different size groups from 22 mm to 34 mm. The effect of the time of day was studied on six normal animals for each hour during different days. The counts for 9 o'clock analysis were taken between 9:00 AM and 9:45 AM; similarly, counts were taken for different hours up to 6 o'clock in the evening. The effect of thermal stress was studied following an earlier report (Ravindranath, 1975a), by subjecting the animals to thermal shock for ten minutes. This was achieved by immersing the animal in sea water maintained at required temperature. All the observations were made betwen May and August, 1975. The statistical analyses carried out in the present study include Student's *t*-test and analysis of variance.

Results

General characters

The granular hemocytes, together with their modified versions such as plasmatocytes and cystocytes (Ravindranath, 1975b), constitute more than 95% of the hemocyte population. In fact, the total hemocyte count (THC) represents primarily the granular hemocytes, as other cell types such as spherule cells and adipohaemocytes are very rare. Spherule cells, dividing granular hemocytes, binucleate and asymmetically dividing granular cells are frequently observed in the afternoon analyses of the hemolymph and also in the hemolymph of postmolt and injured intermolt animals.

TABLE I

Total counts and distribution of hemocytes of Emerita asiatica *in the squares of the hemocytometer.*

Blood Drop	Upper Corner		Central	Lower		THC mm³	Difference between first and subsequent drops	Difference between highest and lowest number of cell among five squares
	1 mm²	2 mm²	mm²	1 mm²	2 mm²			
First	715 (21.14)	661 (19.8)	724 (21.69)	619 (18.54)	619 (18.54)	6,676	338	105 (3.15)
Subsequent	617 (19.78)	698 (22.02)	636 (20.06)	611 (19.28)	607 (19.15)	6,338		91 (2.87)
First	1,184 (21.35)	1,104 (19.89)	1,135 (20.46)	1,058 (19.06)	1,064 (19.18)	11,090	2,056	126 (2.29)
Subsequent	976 (21.06)	934 (20.67)	905 (20.03)	902 (19.97)	800 (17.71)	9,034		176 (3.89)
First	886 (21.89)	819 (20.24)	846 (20.90)	745 (18.41)	747 (18.46)	8,086	374	141 (3.48)
Subsequent	790 (20.45)	833 (21.60)	749 (19.42)	742 (19.24)	742 (19.24)	7,712		91 (2.36)
First	925 (21.16)	977 (22.35)	880 (20.15)	801 (18.32)	788 (18.02)	8,742	748	189 (4.33)
Subsequent	882 (22.06)	791 (19.89)	830 (20.76)	762 (19.05)	732 (18.31)	7,994		150 (3.75)
First	900 (21.40)	894 (21.20)	849 (20.20)	794 (20.20)	765 (18.88)	8,404	1,400	135 (3.18)
Subsequent	761 (21.73)	770 (22.27)	709 (20.25)	652 (18.62)	610 (17.70)	7,004		160 (5.03)

(Numbers in parenthesis are percentage values.)

Distribution of hemocytes in the hemocytometer

Table I shows the patern of distribution of hemocytes in the squares of the chamber, when the chambers are filled in single capillary action with fresh, undiluted hemolymph. The number of cells present in the squares are not uniform. In the corner squares that are toward the central ridge, there are always more cells than in squares toward the side of entry of blood. Although the numbers in different squares are unequal, the central square always contained about 20% of the total cell distributed in five squares. Statistically, the number of cells in the central square is not significantly different from the mean number of cells distributed in all five squares. An interesting feature pertaining to THC noticed in the present study is the difference in the number of cells present in the first drop and subsequent drops. From observations made on 41 crabs, it is noted that the first drop yielded an average count of 8185 ± 2212 cells/mm³, which is higher than the number obtained from the second drop of hemolymph (7451 ± 2410 cells/mm³). The cause for such differences between first and subsequent drops has been suggested to be due to adhesion of cells to the wounded sites (Wigglesworth, 1956; Matsumoto and Sakurai, 1956). Size and time of day do not influence in any way the difference in the number of cells between first and subsequent drops.

THC in mature females

The magnitude of hemocyte counts in normal mature females show a fairly wide range. The THC values presented in this study are the average of the values of first and subsequent drops. The THC varies from 3723 cells to 14,185 cells/mm³, the mean being 7891 ± 1778 cells/mm³.

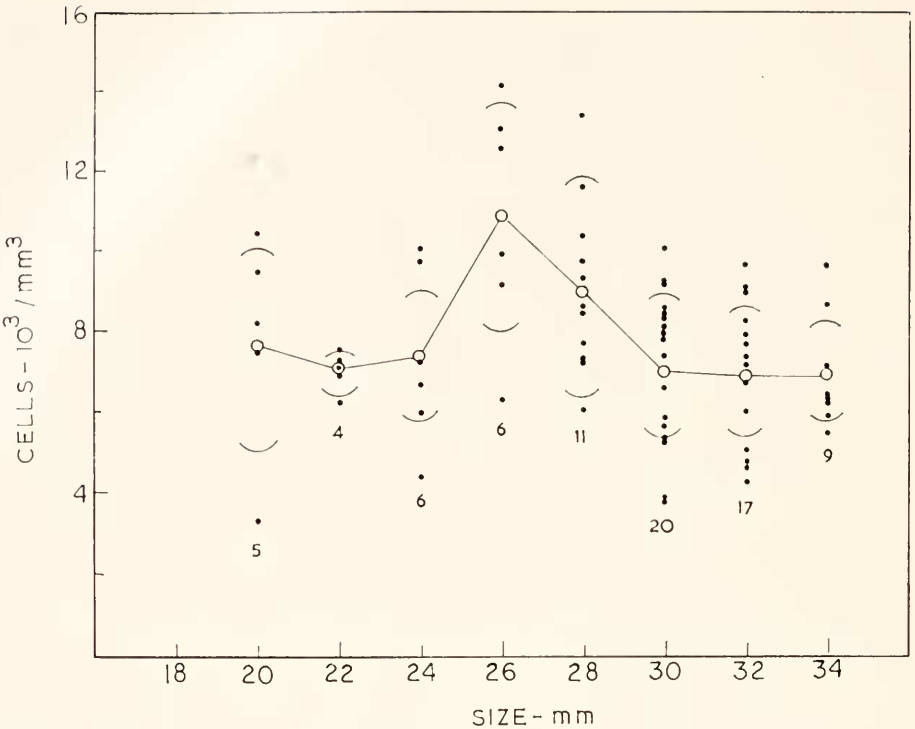

FIGURE 1. Variation in numbers of circulating hemocytes in relation to size for the mole-crab, *Emerita asiatica* (intermolt females). Mean values for each size group are shown as circles. The numbers given below the mean values are each sample size. Parentheses above and below the mean values show standard deviations.

Size as a factor influencing THC

The wide variations in THC's reported above could be due to differences in size (Fig. 1). The count is higher in animals belonging to size groups 26–28 mm than in size groups 22–24 mm, and 30–34 mm. The mean value in the size groups 30 mm, 32 mm, and 34 mm remains somewhat constant.

The variations observed in THC's in different size groups could be due to differences in the degree of dilution of the hemolymph. The water content in the hemolymph of crabs belonging to the size group 22–26 mm range from 92.7% to 95.05% with a mean of 93.72%. For size group 30–34 mm, the water content ranges from 94.21 to 92.57 with a mean of 92.94%, indicating that the water content does not differ from that of the former group. It is obvious that the observed variations in the THC in different size groups may be due to some factors associated with the age of the individuals. What is interesting in this connection is that even among size groups 30 mm, 33 mm and 34 mm, the THC varied from 3723 cells/mm^3 to 10,062 cells/mm^3. It may be recalled that the reproductive stage of the animal (ovigerous females)in this size range is the same and the blood water content is also constant in these animals.

Time of day as a factor influencing THC

Figure 2 shows that time of day could influence the THC. The THC of forenoon analyses are somewhat lower than afternoon analyses. It may be seen that the value of THC is low during 11:00 to 11:45 AM (5750 ± 1534 cells/mm^3) and increases to 9638 ± 2628 during 3:00 to 3:45 PM. This value subsequently declines steadily.

Effect of thermal stress on THC

Data obtained in the present study also show that thermal stress (either heat or cold) to animals, at a period when counts are minimal, may drive the hemocytes into circulation. THC at 20° C ranges from 6403 cells/mm^3 to 9610 cells/mm^3 with a mean of 7718 ± 1212 cells/mm^3. This value is significantly higher than that of animals analyzed at 30° C. THC at 30° C ranges from 3723 cells/mm^3 to 7648 cells/mm^3, with a mean of 5751 ± 1594 cells/mm^3. Heat treatment at 40° C reduces the count to a mean of 3598 ± 1447 cells/mm^3 with a range of 1936 cells/mm^3 to 5747 cells/mm^3. The average water content of the hemolymph of six animals subjected to thermal stress at 20° C, 30° C, and 40° C is 91.5% and 93.5% and 92.0%, respectively.

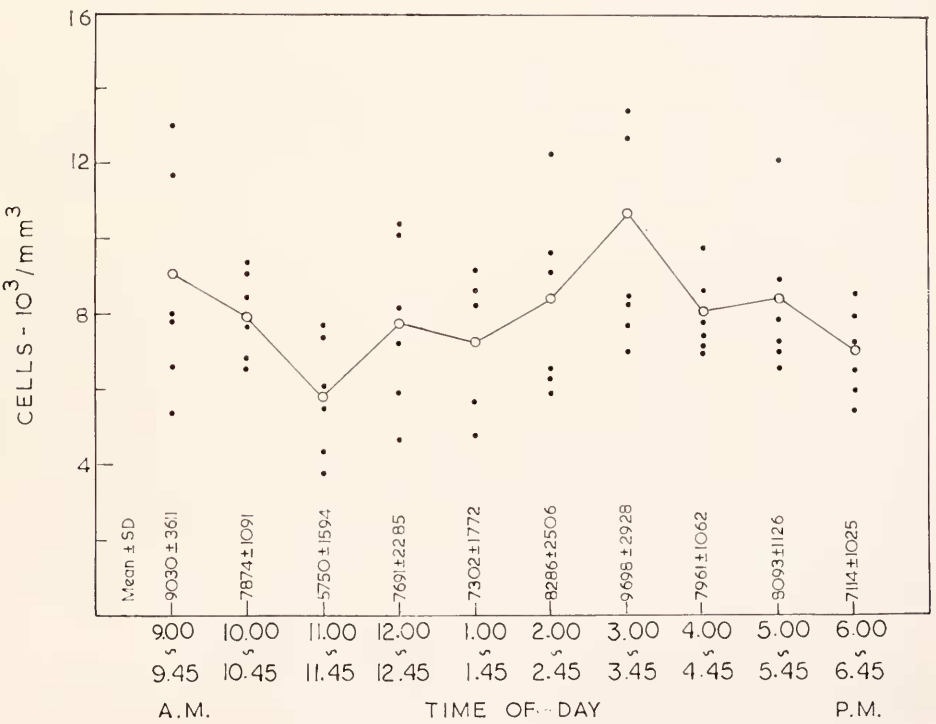

FIGURE 2. Variation in numbers of circulating hemocytes in relation to time of day. The mean values for each size group are shown as circles. The sample size analyzed each hour is six in all cases.

Discussion

The normal count of circulating hemocytes of *Emerita asiatica* was determined after minimizing the inaccuracies caused due to techniques. Errors in counts caused by diluents were avoided. Use of glass pipettes, to which cells adhere (Garvin, 1961), was avoided. The capillary force with which the fresh blood filled the chamber facilitated good distribution and settlement of cells and reduced the magnitude of the field error (see Table I). During the course of this study a new problem arose. As a result of differences between first and subsequent drops used to fill both chambers of the hemocytometer, the question became which one of these values should be used to establish a normal count. Under a similar situation in an insect, Feir (1964) preferred to use the second drop (although it was approximately half the number obtained from the first drop of hemolymph) because it did not show a wide range like that of first drop. In *Emerita asiatica*, both the first and subsequent drops showed a similar range. Therefore an average of both counts was taken.

One of the possible reasons for the wide range in total hemocyte counts of female intermolt *Emerita asiatica*, even after minimizing the technical and observational sources of errors, could be the size of the animals (Fig. 2). Even after controlling the size, the population of hemocytes showed fluctuation in relation to time of day. Although it would be necessary to do sequential sampling over an extended period of time to confirm whether or not there is any daily rhythm in the THC variations, the results suggest possible redistribution of circulating hemocytes during time of day. Such variations in the hemocyte population may occur as a result of the action of any one or a combination of the following mechanisms: alterations in the water content of the blood; alterations in the rate of production of cells; alteration in the rate of destruction of cells; or redistribution of cells in circulation.

The observed variations in THC in different size groups and in relation to time of day may not be due to any change in the concentration of hemolymph, since hemolymph water content did not show significant variation under these conditions. The variations could possibly be due to differences in the rate of production or destruction or hemocytes. In this connection, the observations of Shapiro (1968) pertaining to the dynamics of hematological equilibrium of the larvae of an insect *Galleria mellonella* is of considerable interest. Shapiro (based on the mean values of heat-fixed data, a procedure known to drive hemocytes into circulation) calculated that 76 mitotic cells were found to form a new hemocyte complex approximately every hour, which would contribute to an increase of 3648 hemocytes after a day. Similarly, he found that 921 hemocytes degenerate per hour, which would amount to destruction of 22,104 cells per day. Yet the actual normal of THC is about 37,000 cells per microliter. If the above assumptions of the author were correct, both the rate of production and destruction of cells could not account for the normal hematological equilibrium; moreover, in the absence of an authentic hemocytopoietic tissue in the larvae (see Jones, 1970), it is reasonable to expect some other mechanism contributing to the maintenance of hematological equilibrium.

The fall in hemocyte count could be due to retirement of hemocytes from circulation. Cells thus retired may adhere to tissues or may be sequestered and stored

or destroyed. Cells stored by tissues may come out and re-enter circulation. Possibly such a mechanism may operate both in *Emerita asiatica* and also in the insect, *Galleria mellonella*. Observations by Jones (1968, as cited in 1970) that loose accumulations of cells are found near the wing discs of *Galleria mellonella* support the above suggestion. A critical perusal of the review of Jones (1970) would also indicate that the so-called hemocytopoietic tissues reported by a number of investigators in most insects (see Jones, 1970) appear to be such an accumulation of hemocytes resulting from retirement of circulating hemocytes rather than authentic hemocytopoietic organs.

The experiments carried out in this study in order to verify whether the low THC that occurs between 11 : 00 AM and 11 : 45 AM is due to retirement of cells from circulation confirm the above suggestion. The results further suggest that environmental conditions may also influence the hematological equilibrium of the animal. It may be noted that in the above conditions the THC does not increase beyond a particular level and falls within the range of THC of the size groups. This feature, together with the fall in THC that occurs in the aging animals (of size groups 30–34 mm), suggests that those hemocytes that retire from circulation in aging animals might be destroyed in tissues to which they adhere, probably after sequestration by tissues, as in vertebrates. Redistribution of circulating hemocytes in tissues reaffirms the earlier suggestion (Ravindranath, 1974) that this mechanism is the basis of many, if not all, of the physiological and pathological variations in the hemocyte population.

The results of this investigation also elucidate some aspects pertaining to the life cycle of hemocytes in *Emerita asiatica*. The life span of hemocytes can now be arbitrarily divided into three phases. First, the phase of hemocytopoiesis, the initiation of development of hemocytes from their stem cell to the times at which they are liberated into circulation; secondly, the circulating phase, in which the hemocyte is in circulation; and thirdly, the noncirculating phase when the hemocyte is out of circulation in the tissue proper. Information available for arthropods is restricted to production of hemocytes. In insects, hemoctyes arise both from hemocytopoietic tissue (Hoffmann, 1970) and also from mitosis of hemocytes in circulation (see Jones, 1970). In several crustaceans [seventeen species of decapods including *Emerita* (= Hippa) *talpodia*], Yeager and Tauber (1935) could not observe more than one mitotic figure in 2000 cells they examined. Although they attributed such low mitotic indices to the presence of leucopoietic tissues in these animals, nothing is known regarding sex, size, nutritional, reproductive or molting conditions of the aniamls investigated. During the present investigation, it was noticed that mitosis is not as rare a feature as reported by the above authors but is restricted to fresh and postmolt animals and possibly to certain times of the day in intermolt ovigerous females. Possibly due to these reasons, mitosis of circulating hemocytes in several arthropods would have been overlooked (see literature cited by Jones, 1970; Ravindranath, 1974).

Furthermore, the present study reveals existence of a noncirculating phase during the life span of hemocytes. Studies made on insects (see Jones, 1970) support the existence of such a phase in the lifespan of hemocytes. This is of interest in all arthropods in view of their open circulatory system.

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Summary

1. The normal count of circulating hemocytes of the mole-crab *Emerita asiatica* was studied by taking into consideration the effects of size, time of the day and thermal stress.

2. Total hemocyte counts were higher in animals belonging to size groups 26–28 mm than in size groups 22–24 mm and 30–34 mm. The water content of hemolymph did not differ among different size groups.

3. Total hemocyte counts also showed variation in relation to time of day. Counts were higher in afternoon than in forenoon. The results suggested a possible redistribution of circulating hemocytes in tissues during various times of the day.

4. The above suggestion was supported by the results of the effect of thermal stress on total hemocytes counts, which revealed that the counts decrease as the temperature increases. The findings are discussed in light of previous studies on hematological equilibrium in invertebrates.

5. The results indicate that the life-span of hemocytes may have three phases: the phase of hemocytopoiesis; the circulating phase; and the noncirculating phase, when the hemocyte is out of circulation.

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